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(71) Applicant: TULARIK, INC. [US/US]; 270 East Grand Avenue, South San Francisco, CA 94080 (US).

(72) Inventor: HOEY, Timothy; 270 East Grand Avenue, South San Francisco, CA 94080 (US).

(74) Agents: BREZNER, David, J. et al.; Flehr, Hohbach, Test, Albritton & Herbert, Suite 3400, Four Embarcadero Center, San Francisco, CA 94111-4187 (US).

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(54) Title: HUMAN TRANSCRIPTION FACTORS AND BINDING ASSAYS

(57) Abstract

The invention provides methods and compositions for identifying pharmacological agents useful in the diagnosis or treatment of disease associated with the expression of a gene modulated by a transcription complex containing at least a human nuclear factor of activated T-cells (hNFAT). The materials include a family of hNFAT proteins, active fragments thereof, and nucleic acids encoding them. The methods are particularly suited to high-throughput screening where one or more steps are performed by a computer controlled electromechanical robot comprising an axial rotatable arm.

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Human Transcription Factors and Binding Assays

INTRODUCTION

Field of the Invention

The field of this invention is human transcription factors of activated T-cells.

Background

Identifying and developing new pharmaceuticals is a multibillion dollar industry in the U.S. alone. Gene specific transcription factors provide a promising class of targets for novel therapeutics directed to these and other human diseases. Urgently needed are efficient methods of identifying pharmacological agents or drugs which are active at the level of gene transcription. If amenable to automated, cost-effective, high throughput drug screening, such methods would have immediate application in a broad range of domestic and international pharmaceutical and biotechnology drug development programs.

Immunosuppression is therapeutically desirable in a wide variety of circumstances including transplantation, allergy and other forms of hypersensitivity, autoimmunity, etc. Cyclosporin, a widely used drug for effecting immunosuppression, is believed to act by inhibiting a calcineurin, a phosphatase which activates certain nuclear factors of activated T-cells (NFATs). However, because of side effects and toxicity, clinical indications of cyclosporin (and the more recently developed FK506) are limited.

Accordingly, it is desired to identify agents which more specifically interfere with the function of hNFATs. Unfortunately, the reagents necessary for the development of high-throughput screening assays for such therapeutics are unavailable.

Relevant Literature

Nolan (June 17, 1994) Cell 77, 1-20 provides a recent review and commentary on molecular interactions of hNFAT proteins. Northrop et al. (June 9, 1994) Nature 369, 497-502 report the cloning of a cDNA encoding human NFATc. McCaffrey et al. (October 29, 1993) Science 262, 750-754 report the cloning of a fragment of a gene encoding a murine NFATp$_1$.

SUMMARY OF THE INVENTION

The invention provides methods and compositions for identifying lead compounds and pharmacological agents useful in the diagnosis or treatment of disease associated with the expression of one or more genes modulated by a transcription complex containing a human nuclear factor of activated T-cells (hNFAT). Several forms of hNFAT are provided including hNFATs designated hNFATp$_1$, hNFATp$_2$, hNFATc, hNFAT3, hNFAT4a, hNFAT4b and hNFAT4c. The invention also provides isolated nucleic acid encoding the subject hNFATs, vectors and cells comprising such nucleic acids, and methods of recombinantly producing polypeptides comprising hNFAT. The invention also provides hNFAT-specific binding reagents such as hNFAT-specific antibodies.

Methods using the disclosed hNFATs in drug development programs involve combining a selected hNFAT with a natural intracellular hNFAT binding target and a candidate pharmacological agent. Natural intracellular binding targets include transcription factors, such as AP1 proteins and nucleic acids encoding a hNFAT binding sequence. The resultant mixture is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the hNFAT selectively binds the target. Then the presence or absence of selective binding between the hNFAT and target is detected. A wide variety of alternative embodiments of the general methods using hNFATs are disclosed. The methods are particularly suited to high-throughput screening where one or more steps are performed by a computer controlled electromechanical robot comprising an axial rotatable arm and the solid substrate is a portion of a well of a microtiter plate.

hNFAT SEQUENCE ID NOS:

hNFATp$_1$	cDNA	SEQUENCE ID NO:1

$hNFATp_1$	protein	SEQUENCE ID NO:2
$hNFATp_2$	cDNA	SEQUENCE ID NO:1, bases 1-356 and 868-3478
$hNFATp_2$	protein	SEQUENCE ID NO:2, residues 220-1021
hNFATc	cDNA	SEQUENCE ID NO:3
hNFATc	protein	SEQUENCE ID NO:4
hNFAT3	cDNA	SEQUENCE ID NO:5
hNFAT3	protein	SEQUENCE ID NO:6
hNFAT4a	cDNA	SEQUENCE ID NO:7
hNFAT4a	protein	SEQUENCE ID NO:8
hNFAT4b	cDNA	SEQUENCE ID NO:7, bases 211-2307 and SEQUENCE ID NO:9
hNFAT4b	protein	SEQUENCE ID NO:8, residues 1-699 and SEQUENCE ID NO:10
hNFAT4c	cDNA	SEQUENCE ID NO:7, bases 211-2307 and SEQUENCE ID NO:11
hNFAT4c	protein	SEQUENCE ID NO:8, residues 1-699 and SEQUENCE ID NO:12

DETAILED DESCRIPTION OF THE INVENTION

The invention provides methods and compositions relating to human NFATs. The subject hNFATs include regulators of cytokine gene expression that modulate immune system function. As such, hNFATs and HNFAT-encoding nucleic acids provide important targets for therapeutic intervention.

hNFATs derive from human cells, comprise invariant hNFAT rel domain peptides (see, Table 1) and share at least 50% pair-wise rel sequence identity with each of the disclosed hNFAT sequences. Invariant hNFAT rel domain peptides include from the N-terminal end of the rel domain, HHRAHYETEGSRGAVKA (SEQUENCE ID NO:2, residues 419-435), PHAFYQVHRITGK (SEQUENCE ID NO:2, residues 470-482), IDCAGILKLRN (SEQUENCE ID NO:2, residues 513-523), DIELRKGETDIGRKNTRVRLVFRVHX$_1$P (SEQUENCE ID NO:13), and PX$_2$ECSQRSAX$_3$ELP (SEQUENCE ID NO:14), where each X_1 and X_2 is hydrophobic residue such as valine or isoleucine, and X_3 is any residue, but preferably glutamine or histidine.

Table 1. hNFAT rel domains

NFATp (SEQ ID NO:2, residues 388-678)
NFATc (SEQ ID NO:4, residues 406-697)
NFAT3 (SEQ ID NO:6, residues 397-686)
NFAT4b/c (SEQ ID NO:8, residues 411-702 and SEQ ID NO:10;
SEQ ID NO:8, residues 411-702 and SEQ ID NO:12)

```
NFATp      IPVTASLPPLEWPLSSQSGSYELRIEVQPKPHHRAHYETEGSRGAVKAPT   50
NFATc      SYMSPTLPALDWQLPSHSGPYELRIEVQPKSHHRAHYETEGSRGAVKASA   50
NFAT3      IFRTSALPPLDWPLPSQYEQLELRIEVQPRAHHRAHYETEGSRGAVKAAP   50
NFAT4b/c   IFRTSSLPPLDWPLPAHFGQCELKIEVQPKTHHRAHYETEGSRGAVKAST   50

NFATp      GGHPVVQLHGYMENKPLGLQIFIGTADERILKPHAFYQVHRITGKTVTTT  100
NFATc      GGHPIVQLHGYLENEPLMLQLFIGTADDRLLRPHAFYQVHRITGKTVSTT  100
NFAT3      GGHPVVKLLGYS-EKPLTLQMFIGTADERNLRPHAFYQVHRITGKMVATA   99
NFAT4b/c   GGRPVVKLLGYN-EKPINLQMFIGTADDRYLRPHAFYQVHRITGKTVATA   99

NFATp      SYEKIVGNTKVLEIPLEPKHNMRATIDCAGILKLRNADIELRKGETDIGR  150
NFATc      SHEAILSNTKVLEIPLLPEHSMRAVIDCAGILKLRNSDIELRKGETDIGR  150
NFAT3      SYRAVVSGTKVLEMTLLPEHNMAANIDCAGILKLRNSDIELRKGETDIGR  149
NFAT4b/c   SQEIIIASTKVLEIPLLPEHNMSASIDCAGILKLRNSDIELRKGETDIGR  149

NFATp      KNTKVRLVFKVHIPESSGRIVSLQTASNPIECSQRSAHELPMVERQDTDS  200
NFATc      KNTKVRLVFKVHVPQPSGRTLSLQVASNPIECSQRSAQELPLVEKQSTDS  200
NFAT3      KNTKVRLVFKVHVPQGGGKVVSVQAASVPIECSQRSAQELPQVEAYSPSA  199
NFAT4b/c   KNTKVRLVFKVHIPQPSGKVLSLQIASIPVECSQRSAQELPHIEKYSINS  199

NFATp      CLVYGGQQMILTGQNFTSESKVVFTEKTTDGQQIWEMEATVDKDKSQPNM  250
NFATc      YPVVGGKKMVLSGHNFLQDSKVIFVEKAPDGHHVWEMEAKTDRDLCKPNS  250
NFAT3      CSVRGGEELVLTGSNFLPDSKVVFIERGPDGKLQWEEKATVNRLQSNEVT  249
NFAT4b/c   CSVNGGHEMVVTGSNFLPESKIIFLEKGQDGRPQWEVEGKIIREKCQGAH  249

NFATp      LFVEIPEYRNKHIRTPVKVNFYVINGKRKRSQPQHFTYHPV           291
NFATc      LVVEIPPFRNQRITSPVHVSFYVCNGKRKRSQYQRFTYLPA           291
NFAT3      LTLTVPEYSNKRVSRPVQVYFYVSNGRRKRSPTQSFRFLPV           290
NFAT4b/c   IVLEVPPYHNPAVTAAVQVHFYLCNGKRKKSQSQRFTYTPV           290
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In addition to the shared rel domains, some hNFATs have smaller regions of sequence similarity on the terminal side of the rel domains. For example, the amino terminal regions of hNFAT 4a, 4b and 4c and hNFATc have several regions of similarity (Table 2). The two largest regions (designated regions A and B in Table 2) contain 23 of 41 and 24 of 45 identical amino acids between the two proteins. hNFATp and hNFAT3 also have similarity to other hNFAT proteins in this region (Table 2). The homology between hNFAT3 and hNFAT 4a, 4b and 4c extends about 25 amino acids upstream of the rel region (designated region C in Table 2).

Table 2. hNFAT regions 5' to the rel domain

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A   NFATc      PSTATLSLPSLEAYRDPS-CLSPASSLSSRSCNSEASSYES   195
    NFAT4      PSRDHLYLPLEPSYRESSLSPSPASSISSRSWFSDASSCES   189
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NFATc (SEQ ID NO:4, residues 152-191)
NFAT4a (SEQ ID NO:8, residues 144-184)

```
NFATc    SPQHSPSTSPRASVTEESWLGAR-----SSRPASPCNKRKYSLNG   272
NFAT4    SPRQSPCHSPRSSVTDENWLSPRPASGPSSSRPTSPCGKRRSSAEV  281
```

NFATc (SEQ ID NO:4, residues 233-272)
NFAT4a (SEQ ID NO:8, residues 236-281)

```
B
NFATc                                   SSRPASPCNKRKYSLNG   272
NFAT3                                   SPRPASPCGKRRYSSSG   275
```

NFATc (SEQ ID NO:4, residues 256-272)
NFAT3 (SEQ ID NO:6, residues 259-275)

```
NFATc    SPQHSPSTSPRASVTEESWLGARSSRP                       272
NFATp    SPRTSPIMSPRTSLAEDSCLGRHSPVP                       239
```

NFATc (SEQ ID NO:4, residues 233-259)
NFATp (SEQ ID NO:2, residues 213-239)

```
C
NFAT3          RKEVAGMDYLAVPSPLAWSKARIGGHSP                396
NFAT4          KKDSCGDQFLSVPSPFTWSKPKPG-HTP                410
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NFAT3 (SEQ ID NO:6, residues 369-396)
NFAT4a (SEQ ID NO:8, residues 384-410)

Nucleic acids encoding hNFATs may be isolated from human cells by screening cDNA libraries for human immune cells with probes or PCR primers derived from the disclosed hNFAT genes. In addition to the invariant hNFAT rel sequences and the 50% pair-wise rel domain identity, cDNAs of hNFAT transcripts typically share substantially overall sequence identity with one or more of the disclosed hNFAT sequences.

The subject hNFAT fragments have one or more hNFAT-specific binding affinities, including the ability to specifically bind at least one natural human intracellular hNFAT-specific binding target or a hNFAT-specific binding agent such as a hNFAT-specific antibody or a hNFAT-specific binding agent identified in assays such as described below. Accordingly, the specificity of hNFAT fragment specific binding agents is confirmed by ensuring non-cross-reactivity with other NFATs. Furthermore, preferred hNFAT fragments are capable of eliciting an antibody capable of specifically binding an hNFAT. Methods for making immunogenic peptides through the use of conjugates, adjuvants, etc. and methods for eliciting antibodies, e.g. immunizing rabbits, are well known.

Exemplary natural intracellular binding targets include nucleic acids which comprise one or more hNFAT DNA binding sites. Functional hNFAT binding sites have been found in the promoters or enhancers of several different cytokine genes including IL-2, IL-4, IL-3, GM-CSF, and TNF-α and are often located next to AP-1

binding sites, which are recognized by members of the fos and jun families of transcription factors. Typically, the AP-1 binding sites adjacent to hNFAT sites are low affinity sites, and AP-1 proteins cannot bind them independently. However, many NF-AT and AP-1 protein combinations are capable of cooperatively binding to DNA. Furthermore, cell-type specificity of cytokine gene transcription is often controlled, at least in part, by the combinations of hNFAT and AP-1 proteins present in those cells. For example, there are different classes of T cells that secrete different sets of cytokines: e.g. TH1 cells produce IL-2 and IFN-γ, while TH2 cells produce IL-4, IL-5, and IL-6. hNFAT binding sites are involved in the regulation of both TH1 and TH2 cytokines. Further, differential expression of the cytokine gene in T cell subsets is controlled the combinatorial interactions of hNFAT and AP-1 proteins.

In addition to DNA binding sites and other transcription factors such as AP1, other natural intracellular binding targets include cytoplasmic proteins such as ankyrin repeat containing hNFAT inhibitors, protein serine/threonine kinases, etc., and fragments of such targets which are capable of hNFAT-specific binding. Other natural hNFAT binding targets are readily identified by screening cells, membranes and cellular extracts and fractions with the disclosed materials and methods and by other methods known in the art. For example, two-hybrid screening using hNFAT fragments are used to identify intracellular targets which specifically bind such fragments. Preferred hNFAT fragments retain the ability to specifically bind at least one of an hNFAT DNA binding site and can preferably cooperatively bind with AP1. Convenient ways to verify the ability of a given hNFAT fragment to specifically bind such targets include in vitro labelled binding assays such as described below, and EMSAs.

A wide variety of molecular and biochemical methods are available for generating and expressing hNFAT fragments, see e.g. Molecular Cloning, A Laboratory Manual (2nd Ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor), Current Protocols in Molecular Biology (Eds. Aufubel, Brent, Kingston, More, Feidman, Smith and Stuhl, Greene Publ. Assoc., Wiley-Interscience, NY, NY, 1992) or that are otherwise known in the art. For example, hNFAT or fragments thereof may be obtained by chemical synthesis, expression in bacteria such as E. coli and eukaryotes such as yeast or vaccinia or baculovirus-based expression systems, etc., depending on the size, nature and quantity of the hNFAT or fragment. The subject

hNFAT fragments are of length sufficient to provide a novel peptide. As used herein, such peptides are at least 5, usually at least about 6, more usually at least about 8, most usually at least about 10 amino acids. hNFAT fragments may be present in a free state or bound to other components such as blocking groups to chemically insulate reactive groups (e.g. amines, carboxyls, etc.) of the peptide, fusion peptides or polypeptides (i.e. the peptide may be present as a portion of a larger polypeptide), etc.

The subject hNFAT fragments maintain binding affinity of not less than six, preferably not less than four, more preferably not less than two orders of magnitude less than the binding equilibrium constant of a full-length native hNFAT to the binding target under similar conditions. Particular hNFAT fragments or deletion mutants are shown to function in a dominant-negative fashion. Such fragments provide therapeutic agents, e.g. when delivered by intracellular immunization - transfection of susceptible cells with nucleic acids encoding such mutants.

The claimed hNFAT and hNFAT fragments are isolated, partially pure or pure and are typically recombinantly produced. As used herein, an "isolated" peptide is unaccompanied by at least some of the material with which it is associated in its natural state and constitutes at least about 0.5%, preferably at least about 2%, and more preferably at least about 5% by weight of the total protein (including peptide) in a given sample; a partially pure peptide constitutes at least about 10% , preferably at least about 30%, and more preferably at least about 60% by weight of the total protein in a given sample; and a pure peptide constitutes at least about 70% , preferably at least about 90%, and more preferably at least about 95% by weight of the total protein in a given sample.

Preferred hNFAT fragments comprise at least a functional portion of the rel domain. There are several different biochemical functions that are mediated by the rel and hNFAT rel-similarity domains: DNA binding, dimerization, interaction with B-zip proteins, interaction with inhibitor proteins, and nuclear localization. Other rel family proteins have been shown to physically interact with AP-1 (fos and jun) proteins (Stein et al., EMBO J. 12, 1993). The rel homology domain is necessary for this interaction and the B-zip region of the AP-1 proteins is involved in this protein-protein interaction. The specificity in the ability of hNFAT and AP-1 family members to interact is related to the tissue specific and cell type specific regulation of gene expression governed by these proteins. The rel and rel-similarity domains also

interact with members of the I–κB family of inhibitor proteins including I–κB-like ankyrin repeat proteins (reviewed in Beg and Baldwin, Genes and Dev., 1993). The C-terminal half or the rel domain is involved the interaction with I–κB. There are 5 related I–κB-like proteins which are characterized by having multiple copies of a 33 amino acid sequence motif called the ankyrin repeat.

The invention provides hNFAT-specific binding agents, methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, hNFAT-specific agents are useful in a variety of diagnostic applications, especially where disease or disease prognosis is associated with immune disfunction resulting from improper expression of hNFAT. Novel hNFAT-specific binding agents include hNFAT-specific antibodies and other natural intracellular binding agents identified with assays such as one- and two-hybrid screens; non-natural intracellular binding agents identified in screens of chemical libraries, etc.

Generally, hNFAT-specificity of the binding target is shown by binding equilibrium constants. Such targets are capable of selectively binding a hNFAT, i.e. with an equilibrium constant at least about $10^4\ M^{-1}$, preferably at least about $10^6\ M^{-1}$, more preferably at least about $10^8\ M^{-1}$. A wide variety of cell-based and cell-free assays may be used to demonstrate hNFAT-specific binding. Cell based assays include one and two-hybrid screens, mediating or competitively inhibiting hNFAT-mediated transcription, etc. Preferred are rapid in vitro, cell-free assays such as mediating or inhibiting hNFAT-protein (e.g. hNFAT-AP1 binding), hNFAT-nucleic acid binding, immunoassays, etc. Other useful screening assays for hNFAT/hNFAT fragment-target binding include fluorescence resonance energy transfer (FRET), electrophoretic mobility shift analysis (EMSA), etc.

The invention also provides nucleic acids encoding the subject hNFAT and hNFAT fragments, which nucleic acids may be part of hNFAT-expression vectors and may be incorporated into recombinant cells for expression and screening, transgenic animals for functional studies (e.g. the efficacy of candidate drugs for disease associated with expression of a hNFAT), etc. In addition, the invention provides nucleic acids sharing substantial sequence similarity with that of one or more wild-type hNFAT nucleic acids. Substantially identical or homologous nucleic acid

sequences hybridize to their respective complements under high stringency conditions, for example, at 55°C and hybridization buffer comprising 50% formamide in 0.9 M saline/0.09 M sodium citrate (SSC) buffer and remain bound when subject to washing at 55°C with the SSC/formamide buffer. Where the sequences diverge, the differences are preferably silent, i.e.or a nucleotide change providing a redundant codon, or conservative, i.e. a nucleotide change providing a conservative amino acid substitution.

The subject nucleic acids find a wide variety of applications including use as hybridization probes, PCR primers, therapeutic nucleic acids, etc. for use in detecting the presence of hNFAT genes and gene transcripts, for detecting or amplifying nucleic acids with substantial sequence similarity such as hNFAT homologs and structural analogs, and for gene therapy applications. Given the subject probes, materials and methods for probing cDNA and genetic libraries and recovering homologs are known in the art. Preferred libraries are derived from human immune cells, especially cDNA libraries from differentiated and activated human lymphoid cells. In one application, the subject nucleic acids find use as hybridization probes for identifying hNFAT cDNA homologs with substantial sequence similarity. These homologs in turn provide additional hNFATs and hNFAT fragments for use in binding assays and therapy as described herein. hNFAT encoding nucleic acids also find applications in gene therapy. For example, nucleic acids encoding dominant-negative hNFAT mutants are cloned into a virus and the virus used to transfect and confer disease resistance to the transfected cells..

Therapeutic hNFAT nucleic acids are used to modulate, usually reduce, cellular expression or intracellular concentration or availability of active hNFAT. These nucleic acids are typically antisense: single-stranded sequences comprising complements of the disclosed hNFAT nucleic acids. Antisense modulation of hNFAT expression may employ hNFAT antisense nucleic acids operably linked to gene regulatory sequences. Cell are transfected with a vector comprising an hNFAT sequence with a promoter sequence oriented such that transcription of the gene yields an antisense transcript capable of binding to endogenous hNFAT encoding mRNA. Transcription of the antisense nucleic acid may be constitutive or inducible and the vector may provide for stable extrachromosomal maintenance or integration. Alternatively, single-stranded antisense nucleic acids that bind to genomic DNA or

mRNA encoding a hNFAT or hNFAT fragment may be administered to the target cell, in or temporarily isolated from a host, at a concentration that results in a substantial reduction in hNFAT expression. For gene therapy involving the transfusion of hNFAT transfected cells, administration will depend on a number of variables that are ascertained empirically. For example, the number of cells will vary depending on the stability of the transfused cells. Transfusion media is typically a buffered saline solution or other pharmacologically acceptable solution. Similarly the amount of other administered compositions, e.g. transfected nucleic acid, protein, etc., will depend on the manner of administration, purpose of the therapy, and the like.

The subject nucleic acids are often recombinant, meaning they comprise a sequence joined to a nucleotide other than that which it is joined to on a natural chromosome. An isolated nucleic acid constitutes at least about 0.5% , preferably at least about 2%, and more preferably at least about 5% by weight of total nucleic acid present in a given fraction. A partially pure nucleic acid constitutes at least about 10%, preferably at least about 30%, and more preferably at least about 60% by weight of total nucleic acid present in a given fraction. A pure nucleic acid constitutes at least about 80%, preferably at least about 90%, and more preferably at least about 95% by weight of total nucleic acid present in a given fraction.

The invention provides efficient methods of identifying pharmacological agents or drugs which are active at the level of hNFAT modulatable cellular function, particularly hNFAT mediated interleukin signal transduction. Generally, these screening methods involve assaying for compounds which interfere with hNFAT activity such as hNFAT-AP1 binding, hNFAT-DNA binding, etc. The methods are amenable to automated, cost-effective high throughput drug screening and have immediate application in a broad range of domestic and international pharmaceutical and biotechnology drug development programs.

Target therapeutic indications are limited only in that the target cellular function (e.g. gene expression) be subject to modulation, usually inhibition, by disruption of the formation of a complex (e.g. transcription complex) comprising a hNFAT or hNFAT fragment and one or more natural hNFAT intracellular binding targets. Since a wide variety of genes are subject to hNFAT regulated gene transcription, target indications may include infection, metabolic disease, genetic disease, cell growth and regulatory disfunction, such as neoplasia, inflammation,

hypersensitivity, etc. Frequently, the target indication is related to either immune dysfunction or selective immune suppression.

A wide variety of assays for binding agents are provided including labelled in vitro protein-protein and protein-DNA binding assay, electrophoretic mobility shift assays, immunoassays for protein binding or transcription complex formation, cell based assays such as one, two and three hybrid screens, expression assays such as transcription assays, etc. For example, three-hybrid screens are used to rapidly examine the effect of transfected nucleic acids, which may, for example, encode combinatorial peptide libraries or antisense molecules, on the intracellular binding of hNFAT or hNFAT fragments to intracellular hNFAT targets. Convenient reagents for such assays (e.g. GAL4 fusion partners) are known in the art.

hNFAT or hNFAT fragments used in the methods are usually added in an isolated, partially pure or pure form and are typically recombinantly produced. The hNFAT or fragment may be part of a fusion product with another peptide or polypeptide, e.g. a polypeptide that is capable of providing or enhancing protein-protein binding, sequence-specific nucleic acid binding or stability under assay conditions (e.g. a tag for detection or anchoring).

The assay mixtures comprise at least a portion of a natural intracellular hNFAT binding target such as AP1 or a nucleic acid comprising a sequence which shares sufficient sequence similarity with a gene or gene regulatory region to which the native hNFAT naturally binds to provide sequence-specific binding of the hNFAT or hNFAT fragment. Such a nucleic acid may further comprise one or more sequences which facilitate the binding of a second transcription factor or fragment thereof which cooperatively binds the nucleic acid with the hNFAT (i.e. at least one increases the affinity or specificity of the DNA binding of the other). While native binding targets may be used, it is frequently preferred to use portions (e.g. peptides, nucleic acid fragments) or analogs (i.e. agents which mimic the hNFAT binding properties of the natural binding target for the purposes of the assay) thereof so long as the portion provides binding affinity and avidity to the hNFAT conveniently measurable in the assay. Binding sequences for other transcription factors may be found in sources such as the Transcription Factor Database of the National Center for Biotechnology Information at the National Library for Medicine, in Faisst and Meyer (1991) Nucleic Acids Research 20, 3-26, and others known to those skilled in this art.

Where used, the nucleic acid portion bound by the peptide(s) may be continuous or segmented and is usually linear and double-stranded DNA, though circular plasmids or other nucleic acids or structural analogs may be substituted so long as hNFAT sequence-specific binding is retained. In some applications, supercoiled DNA provides optimal sequence-specific binding and is preferred. The nucleic acid may be of any length amenable to the assay conditions and requirements. Typically the nucleic acid is between 8 bp and 5 kb, preferably between about 12 bp and 1 kb, more preferably between about 18 bp and 250 bp, most preferably between about 27 and 50 bp. Additional nucleotides may be used to provide structure which enhances or decreased binding or stability, etc. For example, combinatorial DNA binding can be effected by including two or more DNA binding sites for different or the same transcription factor on the oligonucleotide. This allows for the study of cooperative or synergistic DNA binding of two or more factors. In addition, the nucleic acid can comprise a cassette into which transcription factor binding sites are conveniently spliced for use in the subject assays.

The assay mixture also comprises a candidate pharmacological agent. Generally a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e. at zero concentration or below the limits of assay detection. Candidate agents encompass numerous chemical classes, though typically they are organic compounds; preferably small organic compounds. Small organic compounds have a molecular weight of more than 50 yet less than about 2,500, preferably less than about 1000, more preferably, less than about 500. Candidate agents comprise functional chemical groups necessary for structural interactions with proteins and/or DNA, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups, more preferably at least three. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the forementioned functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof, and the like. Where the agent is or is encoded by a transfected nucleic acid, said nucleic acid is typically DNA or RNA.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means. In addition, known pharmacological agents may be subject to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc., to produce structural analogs.

A variety of other reagents may also be included in the mixture. These include reagents like salts, buffers, neutral proteins, e.g. albumin, detergents, etc. which may be used to facilitate optimal protein-protein and/or protein-nucleic acid binding and/or reduce non-specific or background interactions, etc. Also, reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, antimicrobial agents, etc. may be used.

The resultant mixture is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the hNFAT specifically binds the cellular binding target, portion or analog. The mixture components can be added in any order that provides for the requisite bindings. Incubations may be performed at any temperature which facilitates optimal binding, typically between 4 and 40 °C, more commonly between 15 and 40°C. Incubation periods are likewise selected for optimal binding but also minimized to facilitate rapid, high-throughput screening, and are typically between .1 and 10 hours, preferably less than 5 hours, more preferably less than 2 hours.

After incubation, the presence or absence of specific binding between the hNFAT and one or more binding targets is detected by any convenient way. For cell-free binding type assays, a separation step is often used to separate bound from unbound components. The separation step may be accomplished in a variety of ways. Conveniently, at least one of the components is immobilized on a solid substrate which may be any solid from which the unbound components may be conveniently separated. The solid substrate may be made of a wide variety of materials and in a

wide variety of shapes, e.g. microtiter plate, microbead, dipstick, resin particle, etc. The substrate is chosen to maximize signal to noise ratios, primarily to minimize background binding, for ease of washing and cost.

Separation may be effected for example, by removing a bead or dipstick from a reservoir, emptying or diluting reservoir such as a microtiter plate well, rinsing a bead (e.g. beads with iron cores may be readily isolated and washed using magnets), particle, chromatographic column or filter with a wash solution or solvent. Typically, the separation step will include an extended rinse or wash or a plurality of rinses or washes. For example, where the solid substrate is a microtiter plate, the wells may be washed several times with a washing solution, which typically includes those components of the incubation mixture that do not participate in specific binding such as salts, buffer, detergent, nonspecific protein, etc. may exploit a polypeptide specific binding reagent such as an antibody or receptor specific to a ligand of the polypeptide.

Detection may be effected in any convenient way. For cell based assays such as one, two, and three hybrid screens, the transcript resulting from hNFAT-target binding usually encodes a directly or indirectly detectable product (e.g. galactosidase activity, luciferase activity, etc.). For cell-free binding assays, one of the components usually comprises or is coupled to a label. A wide variety of labels may be employed - essentially any label that provides for detection of bound protein. The label may provide for direct detection as radioactivity, luminescence, optical or electron density, etc. or indirect detection such as an epitope tag, an enzyme, etc. The label may be appended to the protein e.g. a phosphate group comprising a radioactive isotope of phosphorous, or incorporated into the protein structure, e.g. a methionine residue comprising a radioactive isotope of sulfur.

A variety of methods may be used to detect the label depending on the nature of the label and other assay components. For example, the label may be detected bound to the solid substrate or a portion of the bound complex containing the label may be separated from the solid substrate, and thereafter the label detected. Labels may be directly detected through optical or electron density, radiative emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, etc. For example, in the case of radioactive labels, emissions may be detected directly, e.g. with particle counters or indirectly, e.g. with scintillation cocktails and counters. The methods are particularly suited to automated high throughput drug screening.

Candidate agents shown to inhibit hNFAT - target binding or transcription complex formation provide valuable reagents to the pharmaceutical industries for animal and human trials.

As previously described, the methods are particularly suited to automated high throughput drug screening. In a particular embodiment, the arm retrieves and transfers a microtiter plate to a liquid dispensing station where measured aliquots of each an incubation buffer and a solution comprising one or more candidate agents are deposited into each designated well. The arm then retrieves and transfers to and deposits in designated wells a measured aliquot of a solution comprising a labeled transcription factor protein. After a first incubation period, the liquid dispensing station deposits in each designated well a measured aliquot of a biotinylated nucleic acid solution. The first and/or following second incubation may optionally occur after the arm transfers the plate to a shaker station. After a second incubation period, the arm transfers the microtiter plate to a wash station where the unbound contents of each well is aspirated and then the well repeatedly filled with a wash buffer and aspirated. Where the bound label is radioactive phosphorous, the arm retrieves and transfers the plate to the liquid dispensing station where a measured aliquot of a scintillation cocktail is deposited in each designated well. Thereafter, the amount of label retained in each designated well is quantified.

In more preferred embodiments, the liquid dispensing station and arm are capable of depositing aliquots in at least eight wells simultaneously and the wash station is capable of filling and aspirating ninety-six wells simultaneously. Preferred robots are capable of processing at least 640 and preferably at least about 1,280 candidate agents every 24 hours, e.g. in microtiter plates. Of course, useful agents are identified with a range of other assays (e.g. gel shifts, etc.) employing the subject hNFAT and hNFAT fragments.

The subject hNFAT and hNFAT fragments and nucleic acids provide a wide variety of uses in addition to the in vitro binding assays described above. For example, cell-based assays are provided which involve transfecting a T-cell antigen receptor expressing cell with an hNFAT inducible reporter such as luciferase. Agents which modulate hNFAT mediated cell function are then detected through a change in the reporter.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Investigation of the antigen inducible expression of the IL-2 gene led to the discovery of the regulatory transcription factor NFAT (Nuclear Factor of Activated T cells) (Durand et al. 1988; Shaw et al. 1988). Like several other transcription factors involved in mediating signal transduction, the activity of NFAT is regulated by subcellular localization. In resting T cells NFAT activity is restricted to cytoplasm; stimulation of the T cell receptor leads to translocation of NFAT to the nucleus. Movement of NFAT to the nucleus is dependent on the activation of the calcium-regulated phosphatase calcineurin (Clipstone and Crabtree 1992). The immunosuppressive drugs cyclosporin and FK506 inhibit the activity of calcineurin, and thereby prevent the nuclear localization of NFAT and subsequent activation of cytokine gene expression (reviewed in (Schreiber and Crabtree 1992).

Activation of the T cell antigen receptor induces two signalling pathways required for IL-2 induction, one is the cyclosporin-sensitive, calcium-dependent pathway and the other relies on the activation of protein kinase C (PKC). Antigenic stimulation of these pathways can be mimicked by treating cells with a calcium ionophore and a phorbol ester. The PKC-inducible activity was found to be mediated by fos and jun proteins (Jain et al. 1992; Northrop et al. 1993). The NFAT binding site in the IL-2 promoter is adjacent to a weak binding site for AP-1 proteins, and NFAT and AP-1 proteins bind cooperatively to this composite element (Jain et al. 1993; Northrop et al. 1993). The transcriptional activation mediated by AP-1 proteins through this site appears to be critical for IL-2 expression in activated T cells. There are several different combinations of fos and jun family members that can interact with NFAT to bind DNA (Boise et al. 1993; Northrop et al. 1993; Jain et al. 1994; Yaseen et al. 1994). Therefore, the composition of the AP-1 complex that interacts with NFAT may vary in different cell types and different stages of T cell activation. NFAT was originally reported to be a T cell specific transcription factor critical for the restricted expression of IL-2 (Shaw et al. 1988). More recently, NFAT activity was detected in B cells (Brabletz et al. 1991; Yaseen et al. 1993; Choi et al. 1994; Venkataraman et al. 1994). This is consistent with the finding that, in

transgenic mice, the major sites of expression of a reporter gene regulated by the IL-2 NFAT/AP-1 site are activated T and B cells (Verweij et al. 1990).

In addition to IL-2, NFAT sites have been discovered in the promoters of several other cytokine genes, including IL-4 (Chuvpilo et al. 1993; Szabo et al. 1993; Rooney et al. 1994), IL-3 (Cockerill et al. 1993), GM-CSF (Masuda et al. 1993), and TNF-α (Goldfeld et al. 1993). Thus, it appears that NFAT proteins are involved in the coordinate regulation of many different cytokines in activated lymphocytes. As with IL-2, most of the NFAT sites in other cytokine promoters are composite elements that also contain AP-1 binding sites (Rao, 1994).

Distinct genes encoding NFAT proteins have now been isolated (Jain et al. 1993; McCaffrey et al. 1993; Northrop et al. 1994; Hoey et al., in press). Two of these genes, designated NFATp and NFATc, encode related proteins that are highly similar to each other within a 290 amino acid domain. This NFAT homology region shares weak sequence similarity with the DNA binding and dimerization domain of the rel family of transcription factors (reviewed in (Nolan 1994). There is evidence that both NFATp and NFATc may be involved in mediating transcriptional regulation in activated T cells. For example, NFATp forms a specific complex on DNA with fos and jun that activates transcription in vitro (McCaffrey et al. 1993). NFATc has been shown to activate IL-2 expression by a cotransfection assay in T cells (Northrop et al. 1994). Furthermore, both proteins appears to be modified by calcineurin (Jain et al 1993; Northrop et al. 1994). In addition to NFATp and NFATc, we have isolated two new members of the human NFAT gene family. We have used these clones to examine the tissue distribution of the different NFAT genes. We have also expressed and purified the DNA binding domains of the NFAT family proteins and investigated their biochemical activities.

Results

1. Cloning of human NFAT genes

cDNA libraries were prepared from Jurkat T cells and human peripheral blood lymphocytes, and screened using a probe derived from the rel similarity region of the murine NFATp gene (McCaffrey et al. 1993). Cross-hybridizing clones were isolated, sequenced, and determined to be derived from 4 distinct genes.

One of the genes isolated in this study is related to the murine NFATp gene (McCaffrey et al. 1993), and another is identical to the NFATc gene (Northrop et al.

1994). We have isolated two classes of NFATp cDNAs which are the result of alternative splicing upstream of the rel domain. One form is similar to the cDNA reported by McCaffrey et al., while the other is alternatively spliced downstream of the rel similarity region; in particular, this form is missing an exon encoding the region near the N-terminus of the protein (SEQUENCE ID NO:1, base pairs 357-867) and has a different initiating methionine (SEQUENCE ID NO:1, base pairs 880-882).

In addition to these previously identified genes, we cloned two novel members of the NFAT gene family, hereby designated as NFAT3 and NFAT4. The NFAT3 sequence was obtained from three overlapping cDNAs spanning 2880 bp, and deduced to encode a protein of 902 amino acids. We obtained three classes of NFAT4 cDNAs that resulted from alternative splicing downstream of the rel homology domain. These three types of cDNAs encode proteins that vary in sequence and length at their C-terminal ends. The three forms are designated NFAT4a, NFAT4b, and NFAT4c. The positions of splice junctions in the coding regions are after proline 699 in NFAT4a and after valine 700 and proline 716 in NFAT4b and NFAT4c.

All of the NFAT genes are at least 65% identical to each other within a 290 amino acid domain. This domain is related to the DNA binding and dimerization domain of the rel family of transcription factors (Nolan 1994; Northrop et al. 1994). Among the different NFAT genes, the N-terminal and central portions of the rel similarity domain are more highly conserved than the C-terminus .

Aside from the strikingly similar rel domains shared by all four NFAT genes, the NFAT family members have smaller regions of sequence similarity on the amino terminal side of the rel domains. The amino terminal regions of NFAT4 and NFATc have several regions of significant similarity. The two largest regions contain 23 of 41 and 24 of 45 identical amino acids between the two proteins. Both of these regions are rich in serine and proline residues. NFATp and NFAT3 also have some similarity to the other NFAT proteins in this region, although it is less extensive than that shared between NFAT4 and NFATc. The homology between NFAT3 and NFAT4 extends about 25 amino acids upstream of the rel similarity region.

2. Expression patterns of the NFAT genes

On the basis of previous reports, expression of NFAT genes was expected to be restricted to lymphocytes (Shaw et al. 1988; Verweij et al. 1990; McCaffrey et al.

1993; Northrop et al. 1994). The expression of each NFAT gene was tested by Northern blot using RNA from sixteen different human tissues. For NFATp, expression of an mRNA approximately 7.5 kb was detected in almost all human tissues. The expression was slightly higher in PBLs and placenta. NFATc expression was also detected at a low level in several different tissues. The NFATc probe hybridized to two bands of approximately 2.7 and 4.5 kb. Surprisingly, the 4.5 kb NFATc transcript was strongly expressed in skeletal muscle. The 2.7 kb mRNA appears to correspond to the previously described NFATc clone (Northrop et al. 1994).

NFAT3 exhibited a very complicated expression pattern with at least 3 major RNA bands between 3 and 5 kb. The major sites of NFAT3 expression were observed outside the immune system. NFAT3 was highly expressed in placenta, lung, kidney, testis and ovary. In contrast, NFAT3 expression was very weak in spleen and thymus and undetectable in PBLs.

NFAT4 was expressed predominately as a 6.5 kb message. Like NFATc it was strongly expressed in skeletal muscle. NFAT4 also displayed relatively high expression in thymus. The probe for the NFAT4 northerns contained the 3' half of the NFAT homology region as well as downstream regions from the NFAT4c class of cDNA. This probe should hybridize to all three classes of NFAT4 transcripts. Only one form is detected in the Northern blots, suggesting that the 4c class is the most abundant transcript.

These results indicate that each of the NFAT genes is expressed in a distinct tissue-specific pattern. Furthermore, none of the NFAT genes are restricted to lymphocytes.

3. DNA binding activity of the NFAT proteins

The rel similarity regions along with a small amount of flanking sequences of each of the four classes of NFAT proteins were expressed in E. coli. Each of the 4 proteins was well expressed and soluble. The proteins were purified to near homogeneity by DNA affinity chromatography (Kadonaga and Tjian 1986). The binding site used for purification was a high affinity NFAT site derived from the IL-4 promoter with the core binding sequence GGAAAATTTT (SEQUENCE ID NO:15) (Rooney et al. 1994).

The binding specificities of the NFAT proteins were tested on two known functional binding sites, the IL-4 promoter NFAT site and the NFAT binding site in the distal antigen response element from the IL-2 promoter (Durand et al. 1988; Shaw et al. 1988). All the proteins were able to bind the IL-4 promoter site. NFATp, NFATc, and NFAT3 recognized this sequence with very similar affinity, while NFAT4 bound this sequence with lower affinity (> 10-fold) than the other three proteins in this assay. NFAT4 protein may have a different optimum binding sequence than the other NFAT proteins.

The same amounts of the four NFAT proteins were tested on the NFAT binding site from the IL-2 promoter. This NFAT site (GGAAAAACTG) (SEQUENCE ID NO:16) has three differences relative to the IL-4 site which make it a weaker site for all four NFAT proteins. The NFAT proteins differ in their ability to recognize this site independently. NFATp had the highest relative affinity for the IL-2 binding site, while NFATc and NFAT3 bound weakly to this site and NFAT4 binding was not detectable in this assay.

The IL-2 NFAT site is part of a composite element that is adjacent to a weak AP-1 site (TGTTTCA) (Jain et al. 1992; Northrop et al. 1993). To determine if there were any differences in the ability of NFAT proteins to interact with AP-1, the four NFAT proteins were tested with AP-1 for binding to the IL-2 site. When tested alone all the NFAT proteins, as well as the AP-1 proteins, bound relatively weakly to the IL-2 composite element. The combination of c-jun and fra1 with each of the four NFAT proteins resulted in highly cooperative DNA binding. In the presence of the AP-1 protein the four NFAT proteins bound to the IL-2 site with very similar affinity. In all cases, jun homodimers were not as effective as jun-fra1 heterodimers in promoting cooperative binding in the gel shift assay. These results indicate that the DNA binding and protein interaction specificity of the NFAT proteins are very similar. Indeed, the interactions of the four NFAT proteins with these AP-1 proteins appear to be identical. NFAT4 did not bind independently to this site, but recognized this site with the same affinity as the other NFAT proteins in the presence of AP-1.

4. Transcriptional activation by the NFAT proteins

Having established that the DNA binding properties of the four NFAT proteins are quite similar, we investigated their transcriptional activation potentials. We used a transient transfection assay into Jurkat T cells to measure the ability of the NFAT

proteins to activate the IL-2 promoter. The IL-2 promoter was chosen because it is a critical regulatory target for NFAT and has at least two functional NFAT binding sites (Randak et al. 1990). Activation of this promoter by antigenic stimulation can be mimicked by treatment with phorbol esters, such as phorbol 12-myristate 13 acetate (PMA), together with ionomycin, a calcium ionophore.

Each of the four NFAT genes was transfected into Jurkat cells, and their ability to activate the IL-2 promoter was tested with various combinations of PMA and ionomycin. Treatment of the cells with PMA plus ionomycin induced strong activation by the endogenous NFAT proteins in Jurkat cells. Transfection of each of the four of the NFAT genes resulted in an additional stimulation the IL-2 promoter between 4- and 8-fold. Activation of the IL-2 promoter by each of the NFAT proteins was dependent on both PMA and ionomycin.

We also tested the ability of NFAT to activate transcription in COS and HepG2 cells using a synthetic reporter gene consisting three copies of an NFAT/AP-1 composite element. Transfection of each of the four NFAT into HepG2 cells resulted in activation of the reporter gene of at least 20-fold in the presence of PMA and ionomycin. In contrast to Jurkat cells, NFAT3 was more potent than the others in the HepG2 transfections, resulting in 140-fold activation. Another difference between the results of HepG2 and Jurkat cells is that the NFAT proteins appeared to activate transcription in the absence of PMA or calcium ionophore.

In COS cells NFAT3 produced a striking 50-fold activation that was observed independently of PMA and ionomycin treatment. NFAT3 was found to stimulate transcription in COS cells much more strongly than the other proteins.

5. NFAT proteins are active as monomers

There are many similar features of the NFAT and rel families of transcription factors. Rel proteins form homo- and heterodimers in solution, and dimerization is required for DNA binding (reviewed in Baeuerle and Henkel 1994). The C-terminal half of the rel homology domain is thought to be involved in mediating dimerization. Since the similarity between NFAT and the rel families extends throughout the 300 amino acid rel domain, and the rel domain of the NF-κB proteins is sufficient for dimer formation, we expected that the NFAT proteins might also be function as dimers. To test this idea we determined the native masses of the NFAT proteins by gel filtration chromatography and glycerol gradient centrifugation. For these

experiments we used the rel similarity regions of NFATp and NFATc that were expressed in E. coli and purified by DNA affinity chromatography. The molecular weights of these proteins are 40.4 and 35.6 kD, respectively. As a control we used purified NF-κB p50 protein that is known to exist as a stable dimer in solution (Baeuerle and Baltimore 1989). The p50 protein is 45.8 kD calculated from its amino acid sequence.

On both the gel filtration column and the glycerol gradient, the NFATp and NFATc rel domains migrated at a position close to their actual molecular weight. Under the same conditions, p50 behaved as species that was larger than its monomer molecular weight. The data from the gel filtration column was used to calculate the Stokes radius of each protein, and the S values were determined by glycerol gradient sedimentation. These two properties were used to calculate the apparent molecular size of the proteins (Siegel and Monty 1966; Thompson et al. 1991). The apparent molecular sizes of the NFATp and NFATc rel domains were determined to be 42 kD and 32 kD respectively. These values are close to the monomer molecular weight for both NFAT proteins. As expected, p50 exhibited an apparent molecular size close to that of a dimer.

After determining that NFAT rel domains were monomers in solution, we then considered the possibility that NFAT proteins might form dimers when bound to DNA. To address this question we carried out gel mobility shift assays with two different sized versions of NFATc translated in vitro (Hope and Struhl 1987). The shorter version contains the rel similarity region and a small amount of flanking residues and is referred to as NFATc-309. This construct is equivalent to the one that was expressed in E. coli. The larger version, NFATc-589, contains additional N-terminal sequences. When expressed individually in a rabbit reticulocyte lysate both versions of NFATc were active and produced protein-DNA complexes with different mobilities. When the two different NFATc proteins were mixed by co-translation the same protein-DNA complexes were apparent and no intermediate species was detectable, as would be expected if the proteins were forming dimers on the DNA. These results suggest that NFAT proteins are capable of sequence-specific DNA binding as monomers.

Methods

1. Isolation of human NFAT clones

Peripheral blood lymphocytes (PBLs) were isolated from 2 units of blood (obtained from Irwin Memorial Blood Bank, San Francisco) by fractionation on sodium metrizoate/polysaccharide (Lymphoprep, Nycomed) gradients. Jurkat T cells were grown in RPMI + 10% fetal bovine serum. Total RNA was isolated from Jurkat cells or peripheral blood lymphocytes according to the Guanidinium-HCl method (Chomczynski and Sacchi 1987). Poly-A+ RNA was purified using oligo-dT magnetic beads (Promega). Random primed and oligo-dT primed libraries were prepared from both Jurkat and PBL RNA samples. The cDNA libraries were constructed in the vector Lambda ZAPII (Stratagene) according to the protocol supplied by the manufacturer. The cDNA was size selected for greater than 1 kb by electrophoresis a on 5% polyacrylamide gel prior to ligation. Each library contained approximately 2 X 10^6 recombinant clones. Each of the four libraries was screened independently under the same conditions.

The probe for the initial library screen was a 372 bp fragment derived by PCR from the C-terminal half of the rel homology domain of the mouse NFATp gene. This region corresponds to amino acids 370 through 496 in the published mNFATp sequence (McCaffrey et al. 1993). The fragment was labeled by random priming and hybridized in 1M NaCl, 50 mM Tris pH 7.4, 2 mM EDTA, 10X Denhardt's, 0.05 % SDS, and 50 µg/ml salmon sperm DNA at 60°C. The filters were washed first in 2X SSC, 0.1% SDS, and then in 1X SSC, 0.1% SDS at 60°C. Hybridizing clones were purified and converted into Bluescript plasmid DNA clones. The DNA sequence was determined using thermal cycle sequencing and the Applied Biosystems 373A sequencer. Approximately 50 clones were isolated from the first set of screens. Sequence analysis and cross-hybridization experiments indicated that these clones were derived from 4 distinct genes. For NFAT4, additional cDNA clones were obtained from a skeletal muscle cDNA library (Stratagene). The 5' ends of the cDNA clones were obtained from a Jurkat cDNA library prepared as described above with gene specific primers for each of the NFAT genes.

2. Northerns

The northern blots with mRNA isolated from human tissues were purchased from Clontech. DNA probes were labeled by random priming and hybridized in 5X SSPE, 10X Denhardt's, 50% formamide, 2% SDS, 100 µg/ml salmon sperm DNA at 42°C. The filters were washed in 2X SSC/0.05% SDS at room temperature, and

subsequently in 0.1X SSC/0.1% SDS at 60°C. For NFATp the probe was 1.2 kb cDNA fragment containing the entire rel similarity region of NFATp. For NFATc, the probe was a 291 nucleotide PCR fragment corresponding to the 3' end of rel similarity region (amino acids 597 to 693 (Northrop et al. 1994). For NFATc, a different set of blots was hybridized with a 0.8 kb cDNA fragment located upstream of the rel domain. The two different NFATc probes produced identical results. For NFAT3, the probe was a 0.6 kb fragment located downstream of the rel similarity region corresponding to the region encoding amino acid 720 through the 3' end of the clone. For NFAT4, the probe was a 1.3 kb cDNA fragment corresponding to residue 549 to 963 from the 4c class of cDNAs.

3. Protein Expression and Purification

E. coli expression vectors for each NFAT protein were constructed in the T7 polymerase expression vector pT7-HMK, which has an eight amino acid heart muscle kinase (hmk) site at the N-terminus. NdeI sites were introduced by PCR using mutagenic oligonucleotides in the coding regions upstream of the NFAT rel domains, and these restriction sites were subsequently used for cloning into pT7-HMK. The sizes of the different proteins (without the hmk sequences) are as follows: NFATp, 353 amino acids (the residues homologous to 185 through 537 according to McCaffrey et al. 1993); NFATc, 309 amino acids (amino acids 408 through 716 according to Northrop et al. 1994); NFAT3, 345 amino acids (residues 400 through 744); NFAT4, 316 amino acids (residues 393 through 708). Proteins were expressed using the T7 polymerase expression system in the strain BL21(DE3) (Studier and Moffat 1986). Expression was induced by addition of 0.4 mM IPTG, and the cultures were shaken for 4 hours at room temperature. The cells were harvested, washed in PBS, resuspended in 0.4 M KCl-HEG (25 mM HEPES pH 7.9; 0.1 mM EDTA; 10% glycerol; 0.2% NP-40; 2 mM DTT, 0.2 mM PMSF, 0.2 mM sodium metabisulfite) and lysed by two cycles of freeze-thawing followed by sonication. The lysate was spun in an SS34 rotor at 10K for 10 min to remove insoluble material. NFAT proteins were purified from the soluble fractions of the extracts by DNA affinity chromatography (Kadonaga and Tjian 1986). The binding site sequence for the affinity resin was from the IL-4 promoter, TACATTGGAAAATTTTATTACAC (SEQUENCE ID NO:17). The DNA was biotinylated on one strand and coupled to avidin agarose beads (Sigma) at a concentration of approximately 1 mg DNA/ml.

Approximately 10 mg of E. coli extracts containing the recombinant NFAT proteins were loaded on 1.5 ml DNA columns equilibrated with 0.1 M KCl-HEG. The columns were washed successively with 0.1, 0.2, and 0.4 M HEG. The specifically bound NFAT proteins were eluted with 1.0 M KCl-HEG.

Fra-1 was expressed in E. coli from the vector pET11 (Novagen). The protein was purified from the soluble fraction to approximately 80% homogeneity by fractionation on heparin-sepharose. c-Jun protein was expressed in E. coli and purified from the insoluble portion of the extract as previously described (Bohmann and Tjian, 1989). The concentrations of the purified proteins were determined by comparing the intensity of coomassie staining with the staining intensity of BSA standards.

4. DNA Binding Experiments

Electrophoretic mobility shift assays were performed with the indicated amounts of proteins in 50 mM KCl, 25 mM HEPES, 0.05 mM EDTA, 5 % glycerol, 1 mM DTT with 1 μg of poly(dI-dC) and 100 ng of BSA. The binding reactions and electrophoresis were carried out at room temperature. The samples were run on a 5% polyacrylamide, 0.5X TBE gel at 200 V.

5. Transfections

The full-length coding regions for each of the NFAT genes were subcloned into the RSV expression vector pREP4 (Invitrogen). The reporter plasmid was pXIL2-Luc (constructed by Jim Fraser). It contains the IL-2 promoter (-326 to +47, as in Durand et al 1988) upstream of the luciferase gene. Approximately 1 X 10^6 Jurkat cells were transiently transfected by lipofection (Lipofectin, Gibco/BRL). Twenty hours after transfection the cells were treated with 25 ng/ml PMA and 2 μM ionomycin, and the cells were harvested 8 hours after induction. Transfection efficiencies were standardized by co-transfection of pRSV-βgal and subsequent determination of βgal activity. Each transfection contained 2 μg of expression vector, 5 μg of luciferase reporter, and 1 μg of βgal plasmid and 10 μl of lipofectin. COS-7 and HepG2 cells were transfected by a modification of the calcium phosphate method (Chen and Okayama 1987). The reporter gene contained three copies of the antigen response element (-286 to -257) upstream of the herpes virus tk minimal promoter (-50 to +28) in the luciferase vector pGL2 (Promega).

6. Gel Filtration Columns and glycerol gradients

Protein samples were run on a 2.4 ml Superdex-200 column using the Pharmacia Smart system. The column was equilibrated with 0.5M KCl-HEG at a flow rate of 80 µl/min. The elution volumes of purified NFATc, NFATp, and p50 were determined relative to those of molecular weight standards. Purified p50 was provided by Zhaodan Cao. The following molecular weight standards (10 µg) were chromatographed on separate runs: thyroglobulin (669 kD), β-amylase (200 kD), BSA (66 kD), carbonic anhydrase (29 kD), and cytochrome c (12 kD). The elution volume (V_e) was converted to K_{av} by the equation, $K_{av} = (V_e - V_o)/V_i$, where V_o is the void volume and V_i is the included volume. The Stokes radii were determined from a plot of $(-\log K_{av})^{1/2}$ vs. the Stokes radii of the standards (Ackers 1970).

The S values were determined by glycerol gradient centrifugation. Five ml 10-30% glycerol gradients were prepared using a Beckman density gradient former. The samples were centrifuged in a SW50Ti rotor at 39,000 rpm for 40 hours. After centrifugation, 200-µl fractions were collected and analyzed by gel electrophoresis and coomassie staining. The S values were determined by their sedimentation positions relative to the standards. Native molecular sizes were determined from the Stokes radii (a), S values (s), and the partial specific volumes (V) by the method of Siegel and Monty using the equation $M = 6\pi Nas/1-V$ (Siegel and Monty 1966, Thompson et al. 1991).

7. References cited in Experimental Section

Ackers (1970) *Adv. Prot. Chem.* **24**:343-446; Baeuerle and Baltimore (1989) *Genes & Dev.* **3**:1689-1698; Baeuerle and Henkel (1994) *Annu. Rev. Immunol.* **12**:141-179; Boise et al. (1993) *Mol. Cell. Biol.* **13**:1911-1919; Brabletz et al. (1991) *Nucl. Acids Res.* **19**:61-67; Chen and Okayama (1987) *Mol. Cell. Biol.* **7**:2745-2752; Choi et al. (1994) *Immunity* **1**:179-187; Chomczynski and Sacchi (1987) *Anal. Biochem.* **162**:156-159; Chuvpilo et al. (1993) *Nucl. Acids Res.* **21**:5694-5704; Clipstone and Crabtree (1992) *Nature* **357**:695-697; Cockerill et al. (1993) *Proc. Natl. Acad. Sci. USA* **90**:2466-2470; Durand et al. (1988) *Mol. Cell. Biol.* **8**:1715-1724; Goldfeld et al. (1993) *J. Exp. Med.* **178**:1365-1379; Grabstein et al. (1994) *Science* **264**:965-968; Hohlfeld and Engel (1994) *Immunol. Today* **15**:269-274; Hoyos et al. (1989) *Science* **244**:457-460; Hope and Struhl (1987) *EMBO J.* **6**:2781-2784; Jain et

al. (1992) *Nature* **356**:801-803; Jain et al. (1993) *Nature* **365**:352-355; Jain et al. (1993) *J. Immunol.* **151**:837-848; Jain et al. (1994) *Mol. Cell. Biol.* **14**:1566-1574; Kadonaga and Tjian (1986) *Proc. Natl. Acad. Sci. USA* **83**:5889-5893; Masuda (1993) *Mol. Cell. Biol.* **13**:7399-7407; McCaffrey et al. (1993) *Science* **262**:750-754; McCaffrey et al. (1993) *J. Biol. Chem.* **268**:3747-3752; Mouzaki and Rungger (1994) *Blood* **84**:2612-2621; Nolan (1994) *Cell* **77**:795-798; Northrop (1994) *Nature* **369**:497-502; Northrop (1993) *J. Biol. Chem.* **268**:2917-2293; Randak (1990) *EMBO J.* **9**:2529-2536; Rooney (1994) *EMBO J.* **13**:625-633; Schreiber and Crabtree (1992) *Immunol. Today* **13**:136-142; Shaw (1988) *Science* **241**:202-205; Siegel and Monty (1966) *Biochim. Biophys. Acta* **112**:346-362; Studier and Moffat (1986) *J. Mol. Biol.* **189**:113-130; Szabo (1993) *Mol. Cell. Biol.* **13**:4793-4805; Thompson et al.(1991) *Science* **253**:762-768; Venkataraman et al. (1994) *Immunity* **1**:189-196; Verweij et al. (1990) *J. Biol. Chem* **265**:15788-15795; Yaseen et al. (1994) *Mol. Cell. Biol.* **14**:6886-6895; and Yaseen et al. (1993) *J. Biol. Chem.* **268**:14285-14293.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

1. Protocol for hNFAT - hNFAT dependent transcription factor binding assay.

A. Reagents:

- hNFAT: 20 µg/ml in PBS.

- Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hr, RT.

- Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 0.25 mM EDTA, 1% glycerol, 0.5 % NP-40, 50 mM BME, 1 mg/ml BSA, cocktail of protease inhibitors.

- ^{33}P hNFAT 10x stock: 10^{-8} - 10^{-6} M "cold" hNFAT homolog supplemented with 200,000-250,000 cpm of labeled hNFAT homolog (Beckman counter). Place in the 4 °C microfridge during screening.

- Protease inhibitor cocktail (1000X): 10 mg Trypsin Inhibitor (BMB # 109894), 10 mg Aprotinin (BMB # 236624), 25 mg Benzamidine (Sigma # B-6506), 25 mg Leupeptin (BMB # 1017128), 10 mg APMSF (BMB # 917575), and 2mM $NaVo_3$ (Sigma # S-6508) in 10 ml of PBS.

B. Preparation of assay plates:

- Coat with 120 µl of stock NF-AT per well overnight at 4 °C.
- Wash 2X with 200 µl PBS.
- Block with 150 µl of blocking buffer.
- Wash 2X with 200 µl PBS.

C. Assay:

- Add 80 µl assay buffer/well.
- Add 10 µl compound or extract.
- Add 10 µl ^{33}P-NFAT (20,000-25,000 cpm/0.3 pmoles/well = $3x10^{-9}$ M final concentration).
- Shake at 25C for 15 min.
- Incubate additional 45 min. at 25C.
- Stop the reaction by washing 4X with 200 µl PBS.
- Add 150 µl scintillation cocktail.
- Count in Topcount.

D. Controls for all assays (located on each plate):

a. Non-specific binding (no hNFAT added)

b. cold hNFAT at 80% inhibition.

2. Protocol for hNFAT - AP1 dependent transcription factor binding assay.

A. Reagents:

- fos-jun heterodimers (junB and fra1): 20 µg/ml in PBS.
- Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hr, RT.
- Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 0.25 mM EDTA, 1% glycerol, 0.5 % NP-40, 50 mM BME, 1 mg/ml BSA, cocktail of protease inhibitors.
- ^{33}P hNFAT 10x stock: 10^{-8} - 10^{-6} M "cold" hNFAT homolog supplemented with 200,000-250,000 cpm of labeled hNFAT homolog (Beckman counter). Place in the 4 °C microfridge during screening.
- Protease inhibitor cocktail (1000X): 10 mg Trypsin Inhibitor (BMB # 109894), 10 mg Aprotinin (BMB # 236624), 25 mg Benzamidine (Sigma # B-6506), 25 mg Leupeptin (BMB # 1017128), 10 mg APMSF (BMB # 917575), and 2mM $NaVo_3$ (Sigma # S-6508) in 10 ml of PBS.

B. Preparation of assay plates:

- Coat with 120 µl of stock fos-jun heterodimers per well overnight at 4 °C.

- Wash 2X with 200 µl PBS.

- Block with 150 µl of blocking buffer.

- Wash 2X with 200 µl PBS.

C. Assay:

- Add 80 µl assay buffer/well.

- Add 10 µl compound or extract.

- Add 10 µl ^{33}P-NFAT (20,000-25,000 cpm/0.3 pmoles/well = $3x10^{-9}$ M final concentration).

- Shake at 25C for 15 min.

- Incubate additional 45 min. at 25C.

- Stop the reaction by washing 4X with 200 µl PBS.

- Add 150 µl scintillation cocktail.

- Count in Topcount.

D. Controls for all assays (located on each plate):

a. Non-specific binding (no hNFAT added)

b. cold hNFAT at 80% inhibition.

3. Protocol for hNFAT-fos-jun dependent transcription factor - DNA binding assay.

A. Reagents:

- Neutralite Avidin: 20 µg/ml in PBS.

- Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hr, RT.

- Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 0.25 mM EDTA, 1% glycerol, 0.5 % NP-40, 50 mM BME, 1 mg/ml BSA, cocktail of protease inhibitors.

- ^{33}P hNFAT 10x stock: 10^{-6} - 10^{-8} M "cold" hNFAT homolog supplemented with 200,000-250,000 cpm of labeled hNFAT homolog (Beckman counter) and 10^{-6} - 10^{-8} M fos-jun heterodimers. Place in the 4 °C microfridge during screening.

- Protease inhibitor cocktail (1000X): 10 mg Trypsin Inhibitor (BMB # 109894), 10 mg Aprotinin (BMB # 236624), 25 mg Benzamidine (Sigma # B-6506), 25 mg Leupeptin (BMB # 1017128), 10 mg APMSF (BMB # 917575), and 2mM $NaVo_3$ (Sigma # S-6508) in 10 ml of PBS.

- Oligonucleotide stock: (specific biotinylated). Biotinylated oligo at 17 pmole/µl, AP1-NFAT site: (BIOTIN)-GG AGG AAA AAC TGT TTC ATA CAG AAG GCG T (SEQUENCE ID NO:18)

B. Preparation of assay plates:

- Coat with 120 µl of stock N-Avidin per well overnight at 4 °C.
- Wash 2X with 200 µl PBS.
- Block with 150 µl of blocking buffer.
- Wash 2X with 200 µl PBS.

C. Assay:

- Add 40 µl assay buffer/well.
- Add 10 µl compound or extract.
- Add 10 µl ^{33}P-NFAT (20,000-25,000 cpm/0.1-10 pmoles/well =10^{-9}- 10^{-7} M final concentration).
- Shake at 25C for 15 min.
- Incubate additional 45 min. at 25C.
- Add 40 µl oligo mixture (1.0 pmoles/40 ul in assay buffer with 1 ng of ss-DNA)
- Incubate 1 hr at RT.
- Stop the reaction by washing 4X with 200 µl PBS.
- Add 150 µl scintillation cocktail.
- Count in Topcount.

D. Controls for all assays (located on each plate):

a. Non-specific binding (no oligo added)

b. Specific soluble oligo at 80% inhibition.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A human nuclear factor of activated T-cells, hNFAT, or fragment thereof having an hNFAT specific binding affinity.

2. A human nuclear factor of activated T-cells or fragment thereof according to claim 1, wherein said hNFAT is $hNFATp_1$ (SEQ ID NO:2).

3. A human nuclear factor of activated T-cells or fragment thereof according to claim 1, wherein said hNFAT is $hNFATp_2$ (SEQ ID NO:2, rsidues 220-1021).

4. A human nuclear factor of activated T-cells or fragment thereof according to claim 1, wherein said hNFAT is hNFATc (SEQ ID NO:4).

5. A human nuclear factor of activated T-cells or fragment thereof according to claim 1, wherein said hNFAT is hNFAT3 (SEQ ID NO:6).

6. A human nuclear factor of activated T-cells or fragment thereof according to claim 1, wherein said hNFAT is hNFAT4a (SEQ ID NO:8).

7. A human nuclear factor of activated T-cells or fragment thereof according to claim 1, wherein said hNFAT is hNFAT4b (SEQ ID NO:8, residues 1-699 and SEQ ID NO:10).

8. A human nuclear factor of activated T-cells or fragment thereof according to claim 1, wherein said hNFAT is hNFAT4c (SEQ ID NO:8, residues 1-699 and SEQ ID NO:12).

9. A nucleic acid encoding a human nuclear factor of activated T-cells or fragment thereof according to claim 1.

10. A method of identifying a pharmacological agent useful in the diagnosis or treatment of disease associated with the expression of a gene, wherein the expression of said gene is modulated by a transcription complex comprising a human nuclear factor of activated T-cells (hNFAT), said method comprising the steps of:

forming a mixture comprising a hNFAT or fragment thereof according to claim 1, a nucleic acid capable of selectively binding said hNFAT, a candidate pharmacological agent, and, optionally, a transcription factor different from said hNFAT or fragment thereof,;

incubating said mixture under conditions whereby, but for the presence of said candidate pharmacological agent, said hNFAT or fragment thereof selectively binds said nucleic acid and/or said hNFAT or fragment thereof, said transcription factor and said nucleic acid form a selectively bound complex;;

detecting the presence or absence of selective binding of said hNFAT or fragment thereof and said nucleic acid and/or said selectively bound complex;;

wherein the absence of said selective binding and said selectively bound complex indicates that said candidate pharmacological agent is lead compound for a pharmacological agent capable of disrupting hNFAT dependent gene expression.

INTERNATIONAL SEARCH REPORT

Inte.. .ional application No.
PCT/US96/03113

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 14/47; C12N 15/12; C12Q 1/68
US CL :530/350; 536/23.5; 435/6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 536/23.5; 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG
search terms: NFAT, human, NFATp, NFATc, NFAT3, NFAT4, assay, transcription factor, binding, agent, compound, drug

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NORTHROP et al. NF-AT components define a family of transcription factors targeted in T-cell activation. Nature. 09 June 1994, Vol. 369, pages 497-502, especially page 497.	1-4, 9
X	WO 94/15964 A1 (DANA-FARBER CANCER INSTITUTE, INC.) 21 July 1994, page 1, abstract; page 6, paragraph 2.	1-3, 9-10
X, P	WO 95/08554 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 30 March 1995, page 1, abstract; page 8, paragraph 2.	1, 4, 9-10
X	WO 95/02053 A1 (SCHERING CORPORATION) 19 January 1995, page 1, abstract; page 29, paragraph 2.	1-4, 6-10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 06 MAY 1996	Date of mailing of the international search report 03 JUN 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer TERRY A. MCKELVEY Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/03113

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	HOEY et al. Isolation of Two New Members of the NF-AT Gene Family and Functional Characterization of the NF-AT Proteins. Immunity. May 1995, Vol. 2, pages 461-472, especially page 461, abstract.	1, 5-9
A, P	HO et al. NFATc3, a Lymphoid-specific NFATc Family Member That Is Calcium-regulated and Exhibits Distinct DNA Binding Specificity. The Journal of Biological Chemistry. 25 August 1995, Vol. 270, No. 34, pages 19898-19907, see entire document.	1-10
A, P	MASUDA et al. NFATx, a Novel Member of the Nuclear Factor of Activated T Cells Family That Is Expressed Predominantly in the Thymus. May 1995, Vol. 15, No. 5, pages 2697-2706, see entire document.	1-10
A	MCCAFFREY et al. Isolation of the Cyclosporin-Sensitive T Cell Transcription Factor NFATp. Science. 29 October 1993, Vol. 262, pages 750-754, see entire document.	1-10